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Experimental Brain Tumors, Dendritic Cells and Immunotherapy

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2008

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Abstract

   Malignant astrocytomas are the most common primary tumors of the adult central nervous system. Surgical resection of tumor mass in combination with radiotherapy and chemotherapy is only palliative and there is a clear need for new and more effective therapeutic strategies.

   The aim of this study was to develop a dendritic cell (DC)-based vaccine for the treatment of experimental brain tumors with the future prospect of translating this treatment into the clinical application. We first demonstrated that the N29 and N32 rat brain tumors closely resemble human glioblastoma multiforme and anaplastic astrocytoma, respectively, and represent relevant models to study the efficacy of new therapeutic modalities. We also found that vaccination with IFN-γ-producing tumor cells led to tumor regression in a fraction of animals in both tumor models. The route of vaccine administration significantly influenced the outcome of the therapy. S.c. immunization with IFN-γ-producing tumor cells was far more effective compared to i.d. injection.

   DCs generated from rat bone marrow progenitor cells exhibited the capacity to take up antigens in an immature state and induce T cell proliferation in a mature state, two functional properties central for the induction of anti-tumor immune response. We tested different antigen preparations and maturation factors in order to establish the optimal conditions for DC activation. Synergistic inhibition of intracerebral tumor growth was observed when rats were vaccinated with a combination of *ex vivo* tumor cell lysate-pulsed and matured DCs and IFN-γ-producing tumor cells. However, we did not observe any benefit of using DC-based vaccines alone regardless of antigen loading or maturation methods compared to immunotherapy with IFN-γ-producing tumor cells.

   In conclusion, we have demonstrated that DC-based vaccines fail to provide protection in a weakly immunogenic brain tumor model but do enhance the anti-tumor immune responses elicited by IFN-γ-producing tumor cells. These findings could be pertinent to other tumor models and other immunotherapeutic modalities and thus have important implications for the development of anti-cancer vaccines.

Key words: Brain tumor, dendritic cell, immunotherapy, interferon-γ

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A doctoral thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These have already been published or are manuscripts at various stages (in press, submitted, or in preparation).

"Logic will get you from A to B, Imagination will take you everywhere."
Albert Einstein

To my parents

CONTENTS

ORIGINAL PAPERS

This thesis is based on the following papers referred to by their Roman numericals:

   I.   *Shorena Janelidze, Daniel Bexell, Wiaam Badn, Johan Bengzon Anna Darabi, Karin Enell Smith, Salina Gunnarsson, Peter Milos, Leif G. Salford, Peter Siesjö, and Edward Visse.* Two rat brain tumor models with characteristics of human glioblastoma and anaplastic astrocytoma. Submitted.

  II.   *Shorena Janelidze, Sofia Eberstål, Anna Darabi, Leif G. Saford, Edward Visse and Peter Siesjö.* Route of immunization determines efficacy of interferon-γ-transduced tumor cell vaccine in rat glioma model. Manuscript.

 III.   *Janelidze S, Enell K, Visse E, Darabi A, Salford LG, Siesjö P.* Activation of purified allogeneic $CD4^+$ T cells by rat bone marrow-derived dendritic cells induces concurrent secretion of IFN-γ, IL-4, and IL-10. Immunol Lett 2005; 101: 193-201.

  IV.   *Shorena Janelidze, Sofia Eberstål, Anna Darabi, Karin Enell Smith, Leif G. Salford, Edward Visse and Peter Siesjö.* Mature dendritic cells enhance anti-tumor immunity elicited by IFN-γ-transduced tumor cells in the weakly immunogenic rat N32 glioma model. Submitted.

ABBREVIATIONS

APC	Antigen-presenting cell
BM	Bone marrow
cDC	Conventional dendritic cell
CLP	Common lymphoid progenitor
CLR	C-type lectin receptor
CMP	Common myeloid progenitor
CNS	Central nervous system
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
Flt3-L	Flt3 ligand
GBM	Glioblastoma multiforme
GEM	Genetically engineered mouse
GFAP	Glial fibrillary acidic protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
HLA	Human leukocyte antigen
i.d.	Intradermal
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IKDC	IFN-γ-producing killer dendritic cell
IL	Interleukin
i.l.	Intralymphatic
ILT	Immunoglobulin-like transcript
i.t.	Intratumoral
i.v.	Intravenous
LC	Langerhans cell
LN	Lymph node
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
NK	Natural killer
NLR	Nod-like receptor
NO	Nitric Oxide
PAMP	Pathogen-associated molecular pattern
pDC	Plasmacytoid dendritic cell

PD-L1	Programmed cell death ligand 1
PGE$_2$	Prostaglandin E$_2$
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
s.c.	Subcutaneous
SLAM	Signaling lymphocyte activation molecule
TAA	Tumor-associated antigen
TCR	T cell receptor
TGF-β	Transforming growth factor β
Th	T helper
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
T*reg* cell	Regulatory T cell
wt	Wild type

INTRODUCTION

Cancer, or malignant neoplasm, encompasses a group of diseases characterized by uncontrolled growth and spread of abnormal cells. The abnormality arises as a result of genetic instability leading to the accumulation of multiple genetic lesions. The process of cancer development follows in a way the principles of Darwinian evolution: under the continuous environmental pressure certain combinations of genetic lesions drive progressive transformation of cells from a normal to a malignant state, the latter having numerous growth advantages. Despite significant progress in the treatment of cancer during the last decades, substantial number of different cancer types, especially those at the late stages of disease, remains incurable. Cancer is a still a leading cause of death worldwide and estimated to continue rising. The incidence rates are age-dependent with considerable geographic, race and gender variations. From the evolutionary perspective, the high prevalence of cancer in humans is explained by mismatch between the genes and phenotypic traits that have become common as a result of natural selection in the past and present environmental and social conditions.

Over the years we have witnessed substantial advances in an effort to dissect the epidemiologic, genetic and molecular mechanisms of cancer origin. The cancer research today is a complex multidisciplinary area that reflects the complexity of the disease itself and drives a similarly complex anti-cancer drug development industry. It seems, however, that we are just at the beginning of the long road in our quest for the "Holy Grail" of cancer science.

ASTROCYTIC TUMORS

Classification and grading

Primary tumors of the central nervous system (CNS) encompass a wide range of neoplasms that originate in the CNS and are classified according to the putative resemblance of their cellular components to normal cells. Astrocytic tumors consisting of cells with histologic features of normal astrocytes are the most frequent neoplasms of CNS with approximately 500 new cases diagnosed every year in Sweden. The malignancy of astrocytomas, similar to other tumors, is described in terms of grades. According to the grading system adopted by the World Health Organization (1), the grade of tumor is established based on histopathological appearance, has a prognostic value and ultimately determines the choice of treatment. Malignant astrocytomas are highly invasive tumors (with the exception of grade I tumors) that are graded using several histological criteria, including nuclear atypia, mitotic activity, microvascular proliferation and/or necrosis. Grade I astrocytomas are typically well-circumscribed tumors with very low proliferation indices. These neoplasms primarily occur in children and young adults and are usually curable after surgical resection (2). Grade II diffuse astrocytomas are characterized by increased cellularity and nuclear atypia predominantly affecting young adults. The median survival time is 6-8 years, with a tendency for progression to more malignant grade III-IV tumors that occurs within 4-5 years after diagnosis (2-4). When compared to diffuse astrocytomas, grade III anaplastic astrocytomas display increased cellularity and more prominent nuclear atypia. However, the appearance of distinct mitotic figures is the decisive diagnostic feature. The progression to grade IV glioblastoma multiforme (GBM) is common; time to progression is approximately 2 years (5). GBM is one of the most malignant human neoplasms accounting for 50-60% of all astrocytic tumors (2). This tumor may develop from less malignant lesions (secondary GBM) but most often arises *de novo* (primary GBM). In addition to nuclear atypia and mitotic figures, histopathological characteristics include microvascular proliferation and/or necrosis (absent in lower grade astrocytic tumors). Similar to anaplastic astrocytoma, GBM is prevalent among adults. The median survival time is less than 12 months, with fewer than 3% of patients surviving up to 3 years (2, 5). Young age is a positive prognostic factor for GBM and all malignant astrocytomas in general.

Molecular pathology

Advances in biotechnology has prompted a great number of studies elucidating molecular mechanisms that underlie the formation and progression of cancer. These

Tumors (most common)	Grade	Nuclear atypia	Mitotic activity	Necrosis and/or vascular proliferation	Median survival
Pilocytic astrocytoma	I	−(X)	−(X)	−	Curable
Diffuse astrocytoma	II	X	−	−	6-8 years
Anaplastic astrocytoma	III	X	X	−	3-4 years
Glioblastoma multiforme	IV	X	X	X	< 1 year

studies revealed the remarkable heterogeneity of genetic and epigenetic alterations within histologically identical astrocytic tumors. In some cases a correlation between specific molecular aberration and survival time was found, suggesting the importance of molecular pathogenesis for the diagnostic and prognostic assessments. Furthermore, the identification of clinically relevant molecular alterations could aid the rational development of new therapies in the future.

Neoplastic cells frequently display abnormalities in molecular pathways regulating cell growth, survival and migration that in turn determine the manifestation of their malignant phenotype. Several excellent review articles describe in detail the molecular pathogenesis of malignant brain tumors that is briefly summarized here (6-8).

The p53 pathway

p53 is a tumor suppressor that coordinates cellular responses to stress through the induction of cell cycle arrest, senescence and apoptosis and thus plays a central role in the maintenance of the DNA integrity. The *TP53* gene is mutated in about 50% of human cancers. The most common are missense mutations. The mutations in the *TP53* gene occur at early stages of glial transformations resulting in the same frequency of the loss of p53 functions in both low and high-grade astrocytomas. More than 60% of grade II, III and grade IV secondary astrocytomas carry *TP53* mutations (3, 9) and some studies have shown negative correlation between the presence of *TP53* mutations and the length of time interval before progression in diffuse astrocytoma (3, 10). However, *TP53* mutations were generally not predictive for longer survival when statistical analysis was adjusted for the patients age (5, 11). The mutations in the *TP53* gene are less frequent in primary GBM (< 30%) (5, 9). The function of the p53 protein can be hampered through

other mechanisms, for example by amplification or overexpression of MDM2 and loss of p14[ARF]. MDM2 binds to and inhibits p53 and also promotes its degradation (12). p14[ARF] on the other hand reverts MDM2-induced p53 degradation through direct binding to MDM2 (13, 14). The overexpression of MDM2 has been observed in more then 50% of primary but in only 11% of secondary GBM(15) while p14[ARF] loss occurs at the same rate in both primary and secondary GBM (50% and 70%, respectively) (16). The *MDM2/p14[ARF]* are dysregulated at lower frequencies in anaplastic astrocytomas (4). Contradictory results have been reported in the studies investigating the prognostic value of alterations in the *p53/MDM2/p14[ARF]* pathway in astrocytic tumors (4, 17-22).

The RB1 pathway

The RB1 pathway controls the transition from G1 into S phase of the cell cycle. The phosphorylation of pRB1 by CDK4/cyclin D complex triggers cell entry into S phase, a process that is inhibited when another member of the RB1 pathway, p16[INK4a], binds to CDK4/cyclin D complex (23). The *RB1* gene is mutated in 15-30% (4, 24, 25) and the *CDK4* gene is amplified in 5-15% of high-grade astrocytomas (4, 25, 26). The *p16[INK4a]* alterations have been described in 28-34% of GBM without differences between primary and secondary tumor variants (16, 25) and in 40% of anaplastic astrocytomas (4). Abnormalities in *RB1/CDK4/ p16[INK4a]* were negatively correlated with the survival in astrocytomas (4, 27).

The epidermal growth factor receptor (EGFR) pathway

EGFR is a family of transmembrane receptor tyrosine kinases that bind to EGF-like growth factors. EGFR has been shown to play an important role in survival, proliferation, differentiation and migration of CNS cells (28). *EGFR* amplification and concomitant overexpression of *EGFR* protein have been observed in about 40% of primary GBM (29, 30) but are uncommon in lower grade astrocytomas and in secondary GBM (9, 11, 31). In addition, EGFR mutations occur in 30-40% of GBM and are often amplified (32, 33). The most common are mutations resulting in surface expression of truncated and constitutively activated EGFRvIII variants. Clinical studies generated conflicting result with respect to the prognostic value of *EGFR* alterations (18) (34, 35).

The PTEN pathway

Once activated, various growth factor receptors dock phosphatidylinositol 3-kinase (PI3K) to the cell membrane. PI3K catalyzes the conversion of phosphatidynositol-4,5-biphosphate (PIP2) to PIP3 that in turn activates the downstream effector molecules including AKT. PTEN is a lipid phosphatase that inhibits the function of PI3K and AKT (36). The PI3K/AKT/PTEN pathway plays an important role in the regulation of cell growth, proliferation, metabolism, angiogenesis and apoptosis (37). Point mutations and amplifications of the *PIK3CA* subunit of PI3K have been detected in a fraction of GBM (38-42). In addition, the progression of astrocyic tumors was accompanied by increased activation of AKT (43). Similar to *EGFR*, the *PTEN* alterations were found in anaplastic astrocytoma but at much lower frequencies than in primary GBM (5-18% and 14-40%, respectively) (11, 40, 44) and were rare in secondary GBM (45). *PTEN* mutations were strongly associated with reduced survival in anaplastic astrocytoma but not in GBM (5, 11). However, several other studies have reported the presence of *PTEN* mutations or *PTEN* mRNA levels to be an independent prognostic factor in GBM patients (46-48).

Conventional and novel therapies for the treatment of malignant astrocytomas

Conventional therapy

The survival of the patients diagnosed with malignant astrocytomas has not improved significantly in recent decades. Surgical resection (when possible) together with radiotherapy is the standard therapeutic approach that is beneficial for the treatment of grade II-III tumors, but it only alleviates the disease-associated symptoms in GBM patients. The failure of the conventional therapies is believed to be partly due to the very infiltrative nature of these tumors making complete surgical resection of tumor mass almost impossible. Radiotherapy after surgical resection has been shown to prolong the survival in anaplastic astrocytoma and GBM patients (49, 50). The benefit of using chemotherapy in conjunction with surgery and radiotherapy was under debate for a long time (51). However, recent randomized clinical trials have shown improved survival in GBM patients when radiotherapy was combined with temozolomide compared to radiotherapy alone (52, 53). A phase III randomized clinical study investigating the efficacy of temozolomide against anaplastic astrocytoma is ongoing (the Radiation Therapy Oncology Group (RTOG) protocol 9813). Despite the clear advantage of using temozolomide, the outcome for high-grade astrocytomas remains very poor.

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Experimental therapeutics

Several experimental therapeutic approaches targeting various aspects of tumorigenesis have been described and successfully applied for the treatment of gliomas in animal models. (Glioma is a collective name for all the brain tumors of glial origin that is often used in the context of animal models). For example, gene therapy has been used to deliver pro-apoptotic or suicide genes (FADD, caspase-6 and caspase-8, HSV-tk/GCV system) specifically into the tumor cells and to correct genetic alterations that are associated with the malignant phenotype (introduction of wild-type p53, suppression of EGFR activity with antisense, etc.) (54). Inhibitors of angiogenesis, immunotherapeutic substances, target-based small molecules and replication competent oncolytic viruses are other treatment paradigms being actively investigated (55-57). Numerous clinical trials that have been initiated based on the success of therapeutic modalities in experimental models failed, however, to show benefit for the treatment of human disease and at best improved the survival in a small subset of patients. The overall ineffectiveness of the currently available standard and experimental therapies underscores the need of developing new and more effective treatment strategies.

Animal models

Animal models provide a useful experimental system to study tumorigenesis and evaluate the efficacy and toxicity of new therapeutic modalities. Nevertheless, in addition to the physiological differences existing between species none of the available animal models fully recapitulates human disease. It is, therefore, important to take into consideration these limitations when attempting to translate preclinical data into clinical applications.

Experimental brain tumor models can be roughly divided into transplantable and genetically engineered mouse (GEM) models based on the tumor induction method. In transplantable models, tumor cells lines derived from spontaneously arising or induced tumors are implanted into syngeneic inbred animals. Alternatively, human tumor cells or cell lines (xenografts) can be injected into immunosuppressed or immunodeficient animals. Although offering the possibility of studying human cells, xenograft models lack two important components that normally influence tumor growth, namely the immune system and a native stromal environment. The distinct advantages of transplantable models in general include reproducibility, predictable growth rates and high penetrance. However, due to the lengthy *in vitro* culture, tumor cell lines used for the induction of transplantable tumors might become histoincompatible with the syngeneic animals. In addition, implanted

tumor cell lines grow very rapidly compared to relatively slow development of human counterparts. Both of these phenomena could lead to the increased immunogenicity of transplantable tumors thus making them more susceptible to the elimination by the immune system. The most commonly used mouse brain tumor cell line, GL261, has been generated from tumors induced by intracerebral injection of 3-methylcholanthrene. The GL261 model has recently been characterized in detail and is apparently only moderately immunogenic (58). Other mouse tumor cell lines, SMA-497 and SMA-560, were derived from astrocytomas spontaneously arising in the VM/DK inbred mouse strain (59). The majority of transplantable rat brain tumors were originally induced by intravenous or transplacental administration of nitrosurea compounds. Three of these models, RG2, F98 and CNS-1, are weakly immunogenic infiltrating tumors reliably representing human high-grade astrocytomas (60-62). Other models, such as 9L gliosarcoma, T9, C6 and avian sarcoma virus-induced RT-2 gliomas, are strongly immunogenic and, therefore, not suitable to study the efficacy of new therapeutic modalities (63).

Compared to transplantable models, GEM models, in which tumors arise spontaneously as a result of the germline or somatic cell genetic manipulations, recapitulate more closely the initiation and progression of human malignancies. Furthermore, GEM models allow to study the causal relationship between specific genetic lesions and tumor formation that could lead to the identification of the initiating mutations and possibly the cells of tumor origin. A number of GEM brain tumor models have been generated during recent years. For example, the development of astrocytomas has been observed as a result of oncogenic $V^{12}Ha$-*ras* or v-*src* kinase expression under control of the glial fibrillary acidic protein (GFAP) promoter (64, 65). GFAP is a differentiation marker of normal as well as neoplastic astrocytes. Likewise, the overexpression of the constitutively active form of EGRF in mouse glial cells lacking *Ink4a-Arf* locus induced tumors with characteristics similar to human gliomas (66). Gene transfer of activated AKT and KRac (using RCAS/tv-a system) to mouse neural progenitor cells but not to differentiated astrocytes caused the formation of GBM-like tumors (67). Additional loss of the *Ink4a-Arf* locus in these animals induced tumor from astrocytes and elevated tumorigenesis from progenitor cells (68). Astrocytomas of different grades have also been observed in mice with simultaneous mutation of two tumor suppressor genes: *Nf1* and *Trp53* (69).

The routine use of GEM models is restricted, however, by a few limitations such as low tumor incidence, long and variable latency of tumor formation, the necessity of advanced imaging technologies and labor intensive breeding procedures.

DENDRITIC CELLS (DCS)

DCs are antigen-presenting cells (APCs) of the immune system that play a central role in the initiation and regulation of adaptive immune responses. DCs originate from bone marrow haemotopoietic stem cells, circulate in the bloodstream and populate virtually all tissues. Within DCs, subsets of a specific phenotype, tissue distribution and function have been described across different species. Despite this heterogeneity DCs share several functional properties that underlie their unique capacity to control immune responses. DCs can internalize, process and present antigens, migrate from the site of antigen encounter to the secondary lymphoid tissues and change the functional state of T cells.

Antigen uptake usually takes place in the peripheral tissues where DCs reside in an immature state. Under steady state conditions, immature DCs carrying self-antigens constitutively migrate to the secondary lymphoid tissues and induce T cell tolerance thus providing a physiological mechanism for the elimination of the self-reactive T cells that have escaped thymic deletion (70). According to the so-called "Langerhans cell paradigm", immature DCs, when exposed to foreign antigens and an inflammatory environment, undergo maturation as they migrate to the secondary lymphoid organs (71). Maturation is a complex process defined by the upregulation of major histocompatibility complex (MHC) class II and costimulatory molecules. Mature DCs present antigens captured in the periphery to antigen-specific T cells thereby initiating adaptive immune responses. The Langerhans cell paradigm needs, however, to be refined in order to accommodate new discoveries in DC biology. It has recently been proposed, for example, that DCs residing in the lymphoid organs can pick up antigens from the peripheral migrating DCs and contribute to the activation or tolerization of T cells (72). Moreover, several studies have shown the induction of T cell tolerance by phenotypically mature DCs (73, 74). To avoid confusion with the terminology, "mature" in this thesis only refers to the phenotype of DCs without any references to their function.

Antigen presentation by DCs

DCs capture exogenous antigens using pinocytosis, phagocytosis or receptor-mediated endocytosis. Pinocytosis enables the internalization of soluble antigens and occurs constitutively in DCs (75). Phagocytosis involves uptake of large particulate antigens initiated by the engagement of specific receptors on the surface of DCs. DCs can phagocytize many types of bacteria, as well as living and dying cells (76-78). DCs also

capture macromolecules through the formation of clathrin-coated membrane vesicles, a process called receptor-mediated endocytosis. DC subsets express different sets of endocytic receptors that could explain their responsiveness or unresponsiveness to specific pathogens (79). Once in endosomal compartments, exogenous antigens are degraded by proteases into peptides that bind to MHC class II molecules (80). The MHC class II-peptide complexes are then transported to the plasma membrane for the presentation to CD4$^+$ T cells.

The endogenous antigens (self- or virus-derived) are processed in the cytosol by proteosomes. Generated peptides are translocated into the endoplasmic reticulum by the specialized TAP transporter where they are loaded into the MHC class I molecules. The MHC class I-peptide complexes are transferred through the Golgi complex to the plasma membrane for the recognition by CD8$^+$ T cells (81).

In addition, DCs have the ability to cross-present exogenous antigens on the MHC class I molecules providing the possibility of generating immune responses against viral infections or neoplastic transformations occurring in cells other then APCs (82). Mechanisms underlying the cross-presentation process are not well defined. Experimental evidence indicates that phagosomes containing captured antigens fuse with the ER vesicles containing components of the MHC class I loading pathway thus forming phagosome-ER hybrid compartment. The antigens are transported from the phagosome-ER hybrid compartment into the cytosol for degradation by proteasomes. The cleaved peptides are then returned back to the phagosome-ER compartment and to the ER as well for the loading into MHC class I molecules (82).

High endocytic capacity is a prominent functional feature of immature DCs (83). Maturation is accompanied by a transient increase in the endocytic capacity (84, 85) followed by a dramatic downregulation of antigen uptake function (75, 86).

DC maturation

DC maturation is triggered by the engagement of the receptors sensing the presence of invading microorganisms and inflammatory mediators. Evolutionary conserved pathogen-associated molecular patterns (PAMPs) are recognized by an array of Toll-like receptors (TLRs). Individual TLRs differ in their ligand specificity, localization and cell distribution pattern. Some of the cell surface TLRs (TLR1, 2, 4, 5, 6) typically recognize bacterial products. Others (TLR3, 7, 8, 9) detect viral nucleic acids and are expressed in the intracellular compartments (87). TLRs are coupled to MyD88-dependent or TRIF-dependent signaling pathways that lead to the activation of NFκB among other

transcriptional factors. NFκB regulates the expression of maturation-associated molecules, chemokines and cytokines by DCs (88). TLRs have also been implicated in the modulation of antigen presenting machinery and the self/non-self discrimination (89).

Another group of receptors expressed by immature DCs, the Nod-like receptors (NLRs), the RIG-I-like receptors (RLRs) and the membrane-associated C-type lectin receptors (CLRs), recognize bacteria, viruses and glycosylated ligands, respectively. These receptors seem to modulate and cooperate with TLRs in shaping the pathogen-induced innate and adaptive immune responses (88, 90).

Although DC maturation is defined by upregulation of MHC class II and costimulatory molecules, it is accompanied by profound changes in cytokine production. Several factors influence the DC maturation process. It has been shown that sustained TLR4 or CD40 signaling is required for the induction of cytokine secretion by DCs. At the same time, a transient exposure to the TLR4 ligand is sufficient to upregulate MHC class II and costimulatory molecules (91, 92). The combined activation of PAMP receptors synergistically enhances cytokine production in both human and mouse DCs (93-95). Both, IFN-γ and CD40L, amplify the TLR-induced interleukin (IL)-12 production, but through different mechanisms. Interestingly, this amplification effect was observed when IFN-γ was given before and CD40L after TLR stimulation (96, 97). Taken together these data suggest that DCs continuously adjust their maturation program to allow the detection and translation of environmental changes. Consequently, mature DCs could display varying cytokine production profiles that would have direct impact on the outcome of DC-T cell interactions.

DC and T cell interactions

DC and T cell interactions begin with the binding of the antigen specific T cell receptors (TCRs) to the cognate MHC-peptide complex. However, activation of naïve T cells requires a second costimulatory signal. Engagement of the TCR without costimulation leads to the induction of T cell tolerance through deletion, anergy or expansion of regulatory T (T*reg*) cell subsets. It is not, therefore, surprising that immature DCs expressing very low levels of costimulatory molecules are involved in the initiation of immune tolerance (98-100). Mature DCs, on the other hand, are equipped with all the necessary machinery to drive the activation of naïve T cells. Furthermore, by modifying the cytokine production profile mature DCs tightly regulate T cell differentiation pathways. For example, DC-derived IL-12 promotes the acquisition of a T helper (Th)1 phenotype by CD4^{+} helper T cells (101), whereas the Jagged family of the Notch ligands can bias towards a Th2

phenotype (102). Th1 cells produce high amounts of IFN-γ and are important for the cell-mediated immune responses against intracellular pathogens and cancer. Th2 cells are characterized by the secretion of IL-4 and play an essential role in the humoral responses against extracellular pathogens. Transforming growth factor (TGF)-β together with IL-6 has recently been shown to induce the development of a novel IL-17-producing Th17 subset that is associated with a number of autoimmune diseases (103). Although there is some evidence of DCs involvement in the differentiation of Th17 cells, the primary mechanisms underlying this process *in vivo* are not fully understood (104, 105).

Mature DCs have long been viewed as immunogenic cells. The notion has been challenged by several observations demonstrating that phenotypically mature DCs can promote tolerance (73, 74, 106). DCs can acquire tolerogenic properties after exposure to various cytokines (74, 107). The effector mechanisms mediating tolerogenic function of mature DCs are not yet defined. Most probably, molecules associated with the tolerogenicity of immature DCs drive the tolerance induction by mature DCs as well, including the signaling lymphocyte activation molecule (SLAM), the programmed cell death ligand-1 (PD-L1), DEC-205 (CD205), the inhibitory receptors of the immunoglobulin-like transcript (ILT3/ILT4) family and indoleamine 2,3-dioxygenase (IDO) (107).

The heterogeneity of DCs and the diversity of DC-induced T cell responses raise the possibility of functional specialization between different DC subsets (108). Still, experimental evidence indicates the remarkable functional plasticity of individual DC populations (109). Additionally, the temporal model of DC maturation where DCs sequentially acquire the Th1 and Th2 priming capacities has been proposed (110). The prevalent view currently is that the final outcome of DC and T cell interactions is determined by multiple factors including the ontogenic origin of DCs, the nature of activating signals and the dynamics of environmental change (111).

Subsets and function

Mouse DCs

Mouse DCs comprise CD11c$^+$MHCII$^+$ conventional DC (cDC) and plasmacytoid DC (pDC) populations. pDCs express B220, Ly6C (cross-reacts with anti-granulocyte antibody Gr-1) and intermediate (int) levels of CD11c (112-114).

Three subsets of resident cDCs are found in the spleen of uninfected mice: CD8$^+$CD4$^-$, CD8$^-$CD4$^-$ and CD8$^-$CD4$^+$ DCs (115). Lymph nodes contain two additional CD8^{int} migratory cDC subpopulations that in case of skin-draining lymph nodes represent emigrated interstitial (dermal) DCs and Langerhans cells (LCs) (116). Because the

functional relevance of CD4 expression on DCs is not known, the splenic DCs are usually referred to as CD8$^+$ and CD8$^-$ cells. The functional specialization of DC subpopulations has clearly been shown for mouse CD8$^+$ and CD8$^-$ DCs. For example, the CD8$^-$ cells phagocytize antigens more efficiently than CD8$^+$ cells (117). CD8$^+$ DCs, on the other hand, are better at the uptake of dead cells and have been implicated in the cross-priming of cytotoxic T cells (118-120). Furthermore, it has recently been shown *in vivo* that CD8$^+$ DCs and CD8$^-$ DCs are biased to MHC class I and MHC class II presentation, respectively (121). In addition, CD8$^+$ DCs mainly trigger Th1 responses, unlike CD8$^-$ DCs that primarily drive Th2 differentiation (122, 123). Interestingly, under specific conditions CD8$^-$ DC subsets can acquire the functional characteristics that are normally ascribed to CD8$^+$ cells and vice versa (124, 125).

The majority of mouse thymic cDCs are CD8$^+$CD4$^-$ cells that play an important role in the negative selection of autoreactive T cells (115, 126, 127). Although a minor fraction of CD8$^-$SIRPα^+ DCs is also identified, its function is at present unclear.

Two subsets of cDCs are present in the mouse skin under steady state conditions: epidermal Langerin$^+$ LCs and dermal DCs (DDCs) (128).

Mouse lymphoid organs and skin contain the pDCs as well (129). The pDCs are characterized by the production of a high amount of type I interferons (IFN) in response to virus infections that activate the cytolytic activity and IFN-γ production in NK cells (130, 131). Mature pDC can present antigens to, expand and differentiate naïve T cells (130, 132, 133). The ability of pDC to direct T cell responses can be modified depending on the nature of the maturation signal and antigen concentration (134, 135). The physiological implications of this functional plasticity, that seems to be common to all DC subsets, remains to be established.

A novel subset of mouse IFN-γ-producing killer DCs (IKDCs) sharing several properties with NK cells has been identified recently (136, 137).

Rat DCs

Rat cDCs are characterized as OX62$^+$MHCII$^+$ cells, although organ-specific OX62$^-$ DC populations have been described (138, 139). Two subsets of cDCs are found in rat lymphoid tissues that express different levels of CD4 and SIRPα (140, 141). The CD4$^+$ and CD4$^-$ DC subsets in the spleen have been shown to induce Th1 and mixed Th1/Th2 responses, respectively (142). CD4$^-$SIRP$^-$ DCs transport apoptotic intestinal epithelial cell bodies to the mesenteric lymph nodes indicating possible involvement of these cells in the maintenance of tolerance to self-antigens (143). Interestingly, the CD4$^-$CD103$^+$

subset of spleen but not lymph node DCs displays a cytotoxic activity toward a range of neoplastic and normal cells that is dramatically downregulated upon maturation (144). The subpopulation MHCII$^+$CD4$^+$OX62$^-$CD11b$^-$ pDC subpopulation producing high levels of type I IFN has been identified in the rat lymphoid organs (145). In general, rat DCs and their functional properties and plasticity are not well studied.

Human DCs

Blood-derived DCs are thus far the best studied human DC populations that are defined as HLA-DR$^+$Lin$^-$ cells. Human circulating DCs are divided into conventional CD11c$^+$CD123$^-$ and plasmacytoid CD11c$^-$CD123$^+$BDCA-2$^+$ populations (146-148). Within the cDC population at least four phenotypically distinct subsets have been described based on the expression of CD1c, BDCA3, CD34 and CD16 molecules (149, 150). CD16$^+$ cDCs display the strongest pro-inflammatory profile in response to TLR ligation, while CD1c$^+$ cells seem to be mainly involved in chemotaxis (151).

In addition to the activation of NK cells (152) and induction of anti-viral T cells responses, human pDCs have been reported to trigger the differentiation of T*reg* cells (153-155). Once again, human pDCs show flexibility in the capacity to elicit Th1 and Th2 response that to a large extent depends on the maturation signal (156-158).

The thymus contains three subset of DCs including CD123$^+$CD45RA$^+$ plasmacytoid cells and CD11c$^+$CD14$^-$ and CD11c$^+$CD14$^+$ conventional DCs. A low expression of costimulatory molecules and spontaneous production of IL-10 point to the possible role of thymic DCs in negative selection (159). In contrast, mature thymic CD11c$^+$ DCs mediate the positive selection of T*reg* cells (160).

Similar to mouse, LCs, DDCs and pDCs are found in human skin. The DDCs can be identified by the expression of DC-SIGN (128).

Origin

The ontogeny of individual DC subsets is not well defined. Existing experimental data indicate the remarkable developmental plasticity of DCs. Both common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs) have been shown to be capable of producing all splenic and thymic subsets of DCs in mice as well as human cDC and pDC (161-163). It was later reported that the potential to form DCs was restricted to Flt3$^+$ populations of CMPs and CLPs (164, 165). An additional subset of CX3CR1$^+$CD117$^+$ CMPs was found to generate spleen-resident cDCs but not pDC in mice (166). Taken together, these studies indicate that the subset commitment occurs

downstream of the CMP or CLP and that CX3CR1^{+}CD117^{+} cells might represent the branching point for pDCs.

The immediate precursors of DCs have been identified in bone marrow, spleen, thymus and skin. The CD11c^{+}B220^{+} cells in the mice bone marrow could give rise to both cDCs (CD8^{+} and CD8^{-}) and pDCs, while CD11c^{+}B220^{-} precursors generated only cDCs (167).

When transferred to irradiated or non-irradiated mice, spleen-derived CD11c^{int}CD 45RA^{lo}CD43^{int}SIRPα^{int}CD4^{-}CD8^{-} cells differentiated into all subtypes of splenic cDC (168, 169). Furthermore, these splenic precursor cells displayed differential expression levels of CD24 with CD24^{high} cells committed to the CD8^{+} DC subset and CD24^{low} cells committed to the CD8^{-} subset (169). The development of CD8^{+} cDCs was observed when the thymus of the irradiated recipient mice was reconstituted with thymic early T cell precursor cells (CD4^{low}CD117^{+}CD44^{+}CD25^{−})(170). The pool of proliferating LC precursors exists in the mouse epidermis (171). However, under inflammatory conditions, Gr-1^{high} blood-circulating inflammatory monocytes have been shown to migrate to the skin and differentiate into Langerhans cells (172). Similarly, human CCR2^{+}CD14^{high} monocytes could generate Langerhans cells under certain *in vitro* conditions (173)(174). The origin of pDC is even less clear and pDC precursors have yet to be identified.

DCs and cancer immunotherapy

Cancer cells accumulate a vast number of genetic and epigenetic changes that result in the expression of neoantigens or self-antigens at abnormal levels. These so called tumor-associated antigens (TAAs) could potentially target cancer cells for the elimination by the immune system. However, tumors evade the immune system through the selective growth of immune-resistant tumor cell variants and/or by active suppression of anti-tumor immune responses. Thus, immunotherapeutic strategies that promote recognition of weekly immunogenic tumor cells and that inhibit tumor-associated immune tolerance are considered as a promising alternative cancer treatment modality. The advantage of using immunotherapy for the treatment of cancer lies in its capacity to specifically recognize tumor cells and to prevent cancer recurrence by initiating long-term immunologic memory.

Given the central role of DCs in initiating and controlling immunity, DC-based vaccines has been used extensively for the treatment of experimental tumors and in clinical trials (175). DCs represent a rare cell population making it difficult to isolate large numbers of cells required for vaccinations. Because of that and to control the conditions of antigen loading and maturation, *ex vivo*-generated DCs are commonly used

in immunotherapeutic trials (176). Mouse and rat DCs are grown from bone marrow progenitors in the presence of GM-CSF with or without IL-4 and Flt3 ligand (Flt3-L) (177-180). Human DCs are frequently produced from blood monocytes in the medium supplemented with of granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4 (176). DCs generated from CD34^{+} haematopoietic progenitor cells or peripheral blood DCs have also been used as vaccines (181, 182).

Numerous protocols for the loading of DCs with TAA have been developed. The use of antigenic peptides is restricted by several limitations such as the need of antigen identification and HLA typing and the narrow repertoire of tumor-specific T cells. As an alternative, DCs can be loaded with TAAs by incubation with tumor cell lysate, apoptotic and necrotic tumor cells or by fusion with live tumor cells (183-186). These cell-based approaches require large amounts of tumor tissue and are associated with the increased risk of autoimmunity. Another antigen loading method involves the transfection of DCs with tumor cell-derived RNA that results in the targeting of multiple tumor epitopes to the MHC class I pathway (187). Transfection of DCs with cDNA appears to be inefficient. Consequently, viral constructs have been used to provide defined antigens to be presented by DCs (188). There are several questions related to the antigen loading process that have to be carefully addressed including the dose of antigen, the efficiency and persistency of antigen expression and the timing of antigen loading in relation to the induction of maturation (189).

Various substances have been used for the DC maturation in animal studies. The advantage of using a combination of different maturation factors has recently been demonstrated (95, 190). Human DCs employed in clinical trials are often matured with a cytokine cocktail consisting of IL-1β, tumor necrosis factor (TNF)-α, IL-6 and prostaglandin E (PGE$_2$) (176). However, other maturation protocols have been shown to induce superior DC activation in terms of IL-12 production and cytotoxic T cell responses (191). New maturation strategies have to be validated in clinical conditions, especially in the context of possible induction of T*reg* cells.

The route of administration is yet another critical parameter that might affect the nature of immune responses elicited by DC-based vaccines. For example, intradermal (i.d.) and intralymphatic (i.l.) DC immunizations give rise to Th1 responses, whereas intravenous (i.v.) injections generate non-polarized T-cell and antibody responses (192). Although subcutaneously (s.c.) administered DCs are routinely used in preclinical and clinical trial, the comparison between s.c. and other routes of DC administration has not been reported. Interestingly, animal studies indicate that the vaccination site determines the DC distribution in lymphoid tissues and expression of homing receptors on T cells (193-195).

In most clinical trials, the initial injection of DCs is followed by boosting vaccinations that are given one week, two weeks or one month apart. Nevertheless, the optimal timing and amount of the booster immunizations have to be established.

Multiple clinical trials utilizing DC-based vaccines have so far demonstrated a very low rate of objective clinical responses that are defined as at least 50% reduction in the sum of the products of the perpendicular diameters of all lesions without the 25% growth of any lesion or the appearance of new lesions (196). According to these criteria only a 8.9% response rate has collectively been observed in melanoma patients from different trials. Still, the response rate for DC-based vaccines was higher compared to other cancer vaccines (197). An equally limited efficacy of DC-based immunotherapy has been reported in brain (discussed later), breast and prostate cancers (196). A more recent phase III clinical trial in advanced melanoma failed to show any benefit of DC vaccine versus standard chemotherapy (198). Despite the absence of a significant clinical improvement, vaccine-induced immune responses that are usually measured as a number of antigen specific cytotoxic T cells in peripheral blood and their ability to produce IFN-γ, have been described in many patients. It is apparent that the generation of cytotoxic T cells is not predictive of the overall vaccine efficacy and that monitoring of anti-tumor immunity should incorporate other variables such as activation of helper T cells, migratory capacity of T cells, T cell infiltration and function at the tumor site.

Few studies have evaluated DC-based vaccines for the treatment of patients with malignant brain tumors. Tumor lysate (199-201), acid-eluted peptides from tumor cells (202, 203), tumor homogenate (204) and whole tumor cells (205) were used to load DCs with TTAs. DC vaccines were mostly injected i.d., in some cases s.c. (200, 202) or i.d. and intratumorally (i.t.) (199, 201). Mature DCs were used in two trials (201, 203). However, spontaneous maturation of DCs was observed as a result of antigen loading (203). Interestingly, Yamanaka *et al*. have reported longer survival in patients treated with mature DCs than in patients receiving immature DCs. Additionally, i.d. and i.t. administration of DCs has been found to improve survival compared to i.d. vaccination alone (201). Overall, DC-based vaccines were well tolerated without any evidence of autoimmunity. Vaccine-induced immune responses were detected in a fraction of patients, including systemic cytotoxicity and increased intratumoral infiltration of T cells (199, 200, 202, 203). Yet, clinical improvement was very limited. (206).

Notwithstanding the discouraging results of the clinical trials, DC vaccines are still considered as an attractive anti-cancer treatment strategy. It is generally believed that a better understanding of DC biology and an optimization of vaccination protocols are essential for the further improvements of DC-based therapy.

THE PRESENT STUDY

Aims
- To provide a detailed description of the N29 and N32 experimental brain tumor models and to investigate the similarities and discrepancies of these models to human malignant gliomas. To explore the possibility of improving the survival of animals with pre-established N29 brain tumors in response to peripheral vaccinations with irradiated interferon-γ-transduced tumor cells (Paper I)
- To study the importance of the immunization route for the efficacy of immunotherapy with interferon-γ transduced tumor cells in the rat N32 model of malignant brain tumors (Paper II)
- To examine the phenotype and functional properties of rat bone marrow-derived dendritic cells related to their possible application for the treatment of cancer (Paper III)
- To test if dendritic cells could amplify the anti-tumor responses induced by irradiated interferon-γ-transduced tumor cells in the rat N32 model of malignant brain tumors. To determine how antigen loading and maturation conditions could affect the efficacy of dendritic cell-based vaccine against intracerebral N32 tumors (Study IV)

General Discussion

The N29 and N32 experimental brain tumor models.

Several experimental mouse and rat models of brain tumors have been developed over the years (63). It would be impossible to generate data related to human disease with the help of these models unless we understand their limitations. The aim of the first article was, therefore, to evaluate how close the N29 and N32 models recapitulate human malignant brain tumors and, consequently, which aspects of brain tumor biology are relevant to study in these models. Additionally, we wanted to test if immunotherapy with IFN-γ-transduced tumor cells (IFN-γ-based immunotherapy) that has been shown to improve survival of rats bearing intracerebral N32 tumors (207), is equally effective against the N29 tumors.

Histopathological examination is routinely performed for the diagnosis of brain tumors in humans. This allows for the determination of tumor type as well as grade of the tumor. Using the same technique we found that on the morphological level the

N29 tumors resembled grade IV astrocytoma or GBM, the most common and the most malignant primary brain tumor, whereas the N32 tumors were similar to grade III anaplastic astrocytoma. The classification and grading of the N32 tumor were established based on the presence of nuclear atypia and mitotic figures. The N29 tumors additionally exhibited necrosis and diffuse infiltration into the normal brain tissue.

Similar to the N32 model, regression of the N29 intracerebral tumors occurred in response to peripheral immunizations with IFN-γ-transduced tumor cells (N29-IFN-γ). Interestingly, the treatment was more effective in the N29 model. In addition, we demonstrated that N29 tumors were lethal in all animals and that immunizations with wild type N29 cells did not inhibit the progression of intracerebral tumors. These results suggest that N29 tumors are weakly immunogenic.

To further characterize the N29 and N32 models, intracerebral tumors and respective tumor cell lines were stained against GFAP and EGFR. GFAP is a marker of normal and neoplastic astrocytes that is frequently expressed in astrocytic brain tumors. It is important to mention, however, that increased malignancy is often associated with the dedifferentiation of tumor cells resulting in GFAP loss (208). For example, the most anaplastic regions of GBM, which is a very heterogeneous tumor, consist of GFAP-negative cells. EGFR is dysregulated in approximately 40% of primary GBM (209). We observed a weak GFAP staining in wild type N29 (N32-wt) and N32 (N32-wt) tumor lines that became undetectable in intracerebral tumors. At the same time, tumor cell lines and intracerebral tumors were negative for EGFR. These results are not very surprising if we take into account the long term *in vitro* culture of the N29 and N32 cells. It is also possible that both cell lines were originally derived from undifferentiated EGFR⁻ tumor cells.

In humans, progressive tumors, including malignant brain astrocytomas, produce immunosuppressive factors, such as TGF-β and nitric oxide (NO), to evade elimination by the immune system. Likewise, TGF-β and NO were detected in culture supernatant from wild type and IFN-γ-transduced (N29-IFN-γ and N32-IFN-γ) rat glioma cells lines. Importantly, the secretion of these molecules by N32-IFN-γ and N32-IFN-γ cells could have negative impact on the outcome of IFN-γ-based immunotherapy. It would be interesting to determine if inhibition of TGF-β and NO in IFN-γ-transduced tumor cells at the immunization site could further improve the survival of animals with implanted intracerebral tumors.

Another mechanism that allows tumor cells to evade the immune system is the inhibition of antigen presenting machinery that in some human cancers involves the abnormal expression of MHC class I molecules (210). The base-line levels of MHC class

I in the healthy CNS unlike other tissues are very low or undetectable. Nevertheless, MHC class I immunoreactivity has been detected in human glioma samples (211). Similarly, N29-wt and N32-wt cells expressed MHC class I. As expected, N29-wt and N32-wt cells were negative for MHC class II, B7.1 and B7.2 molecules. Transduction with IFN-γ upregulated MHC class I and II in both N29 and N32 cell lines but had no effect on the B7 expression.

In the course of the IFN-γ-based immunotherapy in order to avoid tumor formation at the immunization site, animals were vaccinated with irradiated N29-IFN-γ or N32-IFN-y cells. We found that irradiated N29-IFN-γ but not N32-IFN-γ cells expressed B7.1. Thus coexpression of B7.1 and MHC molecules could provide a strong immunostimulatory signal that in turn might be responsible for more effective anti-tumor immune responses in the N29 model compared to the N32 model.

Radiation induced apoptosis was detected in wild type and IFN-γ-transduced cell lines albeit with varying kinetics. In contrast, human glioma cell lines frequently show deficiencies in the apoptotic cascade (212). Although apoptosis is generally considered as a non-inflammatory form of cell death, our findings, in agreement with data published by others, indicated that under certain condition apoptotic cells could be strongly immunogenic.

In summary, the N29 and N32 experimental rat brain tumors closely resemble human high-grade astrocytomas and are relevant models to study certain aspect of tumor biology and to test the efficacy of new therapeutic modalities.

Immunization route determines the efficacy of the N32-IFN-γ cell vaccine

Modified whole cell tumor vaccines have often been used in animal models of cancer. In most cases, modification involves addition of factors (adjuvants, cytokines) that enhance the immunogenicity of tumor cells. Modified tumor cells are usually administered s.c. or i.p. probably because these administration procedures are easy to perform and inflict less suffering in the animals. However, to our knowledge no studies have examined or at least reported the importance of the immunization site for the efficacy of whole cell-based vaccines. On the other hand, immune responses induced by the DC vaccines have been shown to be influenced by the administration route (192). In this study, we found better survival of tumor-bearing animals after s.c. compared to i.d. immunizations with IFN-γ-producing tumor cells.

To investigate the mechanisms behind the superior efficacy of the s.c. route, vaccine-elicited changes in the draining lymph nodes (LNs) were analysed. In tracing experiments,

we found s.c. injected black ink mostly in the popliteal LNs and i.d. injected ink in the inguinal LNs. Furthermore, s.c. and i.d. immunizations with N32-IFN-γ-producing cells caused preferential expansion of popliteal and inguinal LNs, respectively. However, we did not observe any significant qualitative or quantitative differences between popliteal and inguinal LN responses, assayed by either cytokine production or proliferation *in vitro*, that could explain the differential outcome of s.c. and i.d. immunization for the treatment of intracerebral N32 tumors. Additional studies examining different LN cell populations (including T*reg* cells) and their proliferation *in vivo* (using BrdU or Ki-67 staining) are warranted.

Two subpopulations of DCs including Langerhans cells and dermal dendritic cells are present in uninflamed skin (128). In contrast, the subcutaneous space contains few DCs and likely depends on infiltration from other tissues or blood. As a result, distinct DC populations could be involved in antigen uptake at s.c. and i.d. immunization sites causing the difference in the immune responses and ultimately in the survival rates. It is also possible that DCs from skin and subcutaneous space induce different patterns of tissue-homing receptors on activated T cells. Also, we cannot exclude that immune responses elicited by vaccination with IFN-γ-producing tumor cells are affected by differential kinetics of IFN-γ release at s.c. and i.d. immunization sites.

The elucidation of these mechanisms will help to clarify whether the results of our study are only relevant to IFN-γ-expressing tumor cells or could be applied to the whole tumor cell-based vaccines in general.

Rat bone marrow-derived dendritic cells

The notion of DC plasticity is exemplified by the ability of specific DC subsets to promote both immunity and tolerance depending on the nature of the maturation signal. This underscores the necessity to strictly control the functional properties of DCs before their application in preclinical or clinical studies. Rat DCs are not well characterized or to be more exact are far less characterized than mouse DCs, which is primarily due to very limited availability of transgenic rat technologies. Nevertheless, our aim was to develop a DC-based therapy in the rat model of brain tumors. The main problem with animal models of cancer is their strong immunogenicity. Consequently, therapies proven effective in these immunogenic models in most cases appear to have little or no benefit for human disease. The N29 and N32 rat models that have been established in our laboratory are only weakly immunogenic and thus suitable to study the efficacy of new therapeutic modalities. But before testing DC vaccines in these models, phenotype and functional properties of rat DCs were characterized.

DCs derived from rat bone marrow progenitors (BMDCs) expressed relatively low levels of OX-62 that is routinely used for the identification of rat DC subsets in lymphoid tissues (139). The number of OX-62 positive cells was lower compared to the values reported by others (178). The discrepancy could be explained by the blocking of Fc receptors in our experiments, which, as we observed, reduces the intensity of OX-62 staining. Rat BMDCs cells exhibited an immature phenotype based on the intermediate levels of MHC class II and low levels of CD80/CD86 expression. BMDCs matured with TLR4 ligand, lipopolysaccharide (LPS), upregulated expression of MHC class II, CD80 and CD86 molecules. Immature BMDCs efficiently internalized FITC-albumin, but their endocytic capacity was dramatically reduced upon maturation. Maturation-related downregulation of endocytosis in rat has not been reported before, although this phenomenon is well described in human and mouse (75).

The induction of effective anti-tumor immunity largely depends on the cell-mediated adaptive immune responses that are driven by Th1 cells. It is important, therefore, that DC-based vaccines trigger proliferation and Th1 differentiation of CD4$^+$ T cells. We found that mature BMDCs induced a strong proliferation of purified allogeneic CD4$^+$ T cells. However, T cells produced IFN-γ and IL-4 suggesting the induction of mixed Th1/Th2 responses. The possible explanation of theses data is that rat BMDCs comprise distinct subpopulations of DCs with varying propensities for T cell differentiation. As a result, LPS-matured BMDCs could initiate both Th1 and Th2 polarization of Th cells.

In addition, IL-10 was detected in the culture supernatants from T cells. IL-10 is an immunosuppressive cytokine known to be produced by Th1 cells to limit immune responses and avoid detrimental damage to the host. DCs can also induce IL-10-secreting T*reg* cells (213). In both cases IL-10 could have a negative impact on the anti-tumor immune responses. Nonetheless, the differentiation and/or expansion of T*reg* cells could be prevented by, for example, modification of DC maturation protocol or by depleting DC subpopulations responsible for the Treg induction.

In summary, rat BMDCs are highly endocytic and stimulate T cell proliferation, but induce mixed rather then polarized differentiation of CD4$^+$ T cells. Further studies are required in order to find the conditions of BMDC generation and maturation optimal for the initiation of anti-tumor immune responses.

Dendritic cell-based immunotherapy of N32 glioma

We have previously demonstrated prolonged survival of tumor-bearing rats vaccinated with IFN-γ-producing tumor cells. The aim of this study was to explore a possibility

of further improving the outcome of immunotherapy in the N32 rat brain tumor model by using a combination of IFN-γ-producing tumor cells and BMDCs. The N32 model was chosen because the efficacy of the IFN-γ-producing tumor cell vaccine was less pronounced here than in the N29 model.

In the first set of experiments, N32-IFN-γ cells were combined with immature BMDCs. Immature DCs co-injected with GM-CSF secreting tumor cells have been shown to generate robust anti-tumor immune responses in an ectopic 9L rat glioma model (214). In this setting, antigen loading and maturation of DCs are thought to occur *in vivo* driven by the the presence of dying tumor cells. Immunizations with immature BMDCs and N32-IFN-γ cells did not, however, augment the survival of animals bearing intracerebral N32 tumors compared to immunization with irradiated N32-IFN-γ cells alone. The inefficiency of immature DCs could have been caused by the failure of the *in vivo* antigen uptake or maturation processes. Therefore, in the next experiments, BMDCs were loaded with the lysate from tumor cells prior to vaccinations. To induce maturation of BMDCs, the TLR7/8 ligand imiquimod was applied to the immunization site. We did not observe any benefit of using a mixture of lysate-pulsed BMDCs, imiquimod and N32-IFN-γ cells compared to N32-IFN-γ cells alone.

Although apoptotic cell death is generally considered to be non-immunogenic, it seems that under certain conditions and in some tumor models DCs loaded with apoptotic bodies elicit strong anti-tumor immune responses. To test if the same applies to the N32 model, BMDCs were loaded with apoptotic N32-IFN-γ cells. We also optimized the maturation of BMDCs using different TLR ligands. The combination of Poly(I:C) and R837 was found to induce the strongest activation of BMDCs, charcterized by the highest levels of MHC class II and IL-12 and the lowest levels of IL-10. IL-12 plays a key role in the differentiation of Th1 cell (215), whereas IL-10 suppresses immune responses through, for example, the generation of T*reg* cells. It should be mentioned that, although statistically significant, the differences in MHC class II levels between BMDCs matured with different TLR ligands were relatively small. Interestingly, vaccination with apoptotic tumor cell-pulsed BMDCs, irradiated N32-IFN-γ cells, poly(I:C) and R837 significantly reduced the survival of tumor-bearing animals compared to immunization with irradiated N32-IFN-γ cells alone.

The viability of *ex vivo* matured rat BMDCs is very low. Therefore, animals were immunized with immature DCs and maturation factors were given in a vaccine instead. Most likely, maturation-coupled BMDC death occurs *in vivo* as well. However, under in *vivo* conditions it could lead to antigen transfer to endogenous DCs, a phenomenon that has been shown to play an important role in the amplification of DC-induced immune

responses (216). Nonetheless, the overall failure of immunotherapy with immature BMDCs raised the possibility that maturation of BMDCs in vivo is somehow hindered by, for example, tumor-derived immunosuppressive factors. To avoid the negative impact of tumor cells, BMDC were pulsed with tumor lysate and matured *ex vivo*. We found that immunizations with matured BMDCs alone were less effective compared to immunizations with N32-IFN-γ cells. However, co-administration of mature BMDCs and irradiated N32-IFN-γ cells synergistically improved the survival of animals bearing intracerebral N32 tumors. Elucidation of the mechanisms underlying this synergy could provide valuable information for the future development of DC-based therapies.

In conclusion, BMDC-based vaccines fail to elicit protective anti-tumor immunity, but do amplify the efficacy of the IFN-γ-producing tumor cells in weakly immunogenic brain tumor model.

Concluding remarks

Despite promising preclinical data, numerous clinical trials have shown very little benefit of using DC vaccines for the treatment of cancer. Based on our present understanding of the functional properties of DCs, the concept of DC-based anti-cancer immunotherapy seems to be valid. It is more likely that the protocols for DC generation, activation and vaccination are flawed. These protocols involve complex multi-step procedures that need to be optimized. The results of our studies indicate that the conditions of antigen loading and DC maturation, as probably many other steps of DC vaccine preparation, could have a critical impact on the efficacy of DC-based immunotherapy of cancer.

The failure of clinical trials can also result from the inability of DC vaccine-induced immune responses to overcome tumor-mediated immune suppression. We must realize that DCs alone might never be sufficient to cure cancer but should rather be combined with other treatments such as, for example, strategies inhibiting tumor-associated immune tolerance.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Glioblastoma multiforme (GBM) är en av de mest aggressiva cancerformerna hos människa och det finns i dagsläget inte någon bot. Dessa tumörer växer på ett diffust och infiltrativt sätt, vilket gör att fullständig kirurgisk avlägsning av tumörmassan är omöjlig. Som ett resultat av detta växer tumören tillbaks hos alla patienter. Patienter som har diagnostiserats med GBM har en väldigt dålig prognos: mindre än 2% överlever 5 år efter diagnos. Därför föreligger det ett stort behov av nya och mer effektiva behandlingsformer.

GBM består, precis som så många andra cancerformer, av en snabbt växande massa bestående av onormala celler. Dessa onormala celler uttrycker molekyler på sin yta, som inte finns hos normala celler. Den huvudsakliga funktionen hos immunsystemet är att eliminera främmande organismer och substanser genom induktion av ett immunsvar. Immunsystemet har förmåga att skilja mellan det som är främmande eller annorlunda från organismens normala tillstånd och kan därför känna igen och avstöta tumörceller. Tumörceller kan dock dämpa ned immunförsvaret genom flertalet mekanismer. Cancer-immunoterapi är ett samlingsnamn för olika behandlingsmetoder som för tillfället är under utveckling. Det slutgiltiga målet med cancer-immunoterapi är att stimulera immunförsvaret till den grad att det blir kapabelt att övervinna tumörframkallad dämpande av immunförsvaret och eliminera tumörceller.

En av de immunterapeutiska metoderna involverar immunisering med så kallade "helcells-baserade-vaccin". I detta fall är tumörceller utvunna från patienters kirurgiskt borttagna tumör(er), modifierade med en adjuvant. Adjuvanter är inom immunologin en substans som förstärker förmågan hos andra substanser att inducera en immunrespons. De adjuvantmodifierade tumörcellerna bestrålas och injiceras sedan tillbaka in i patienten. Bestrålningen är nödvändig för att förhindra tumörcellernas delning och därmed bildandet av ny tumörtillväxt vid injektionsstället. På grund av närvaron av ett adjuvant är immunsvaret, frambringat av adjuvantmodifierade tumörceller, starkt nog att döda tumörceller. Detta gäller inte bara tumörceller vid injektionsstället men även tumörceller som inte kunde tas bort genom kirurgi. Till följd av detta är tumöråterväxt förhindrad. Vår hypotes var att "helcells-baserade vaccin" kunde vara användbara vid behandling av GBM. Som adjuvant ville vi använda interferon (IFN)-γ på grund av dess väl kända immunostimulerande effekter. Nya anti-cancer behandlingsformer är vanligtvis testade på försöksdjur. Eftersom cancer är väldigt ovanligt bland försöksdjur måste det framkallas. Djur med sjukdomar som liknar människors hälsotillstånd kallas djurmodeller. Innan man kan testa effektiviteten hos en ny behandlingform på en djurmodell är det viktigt att man

förvissar sig om att den givna modellen är snarlik sjukdomen hos människa. Därför, i första delen av denna avhandling karaktäriserar vi två råttmodeller av malignt gliom. Vi visar att dessa modeller efterliknar GBM hos människa i många aspekter inom tumörbiologin. Vi upptäckte också att en fraktion av djur med gliom botades efter immunisering med IFN-γ-modifierade tumörceller. För att vidare förbättra resultatet av behandlingen ville vi hitta en behandling som är mer effektiv än IFN-γ-modifierade tumörceller.

Dendritiska celler är specialiserade immunceller som initierar och reglerar immunsvaret. För det första, för att en induktion av ett effektivt immunsvar ska ske, så måste dendritiska celler först ta upp tumörcellsfragment, klyva dem till små peptider och uttrycka dessa peptider på sin yta. Denna process kallas antigenpresentation. För det andra måste en ordentlig utmognad av de dendritiska cellerna ske, eftersom omogna dendritiska celler har en immunhämmande funktion. Experimentella data indikerar att en av mekanismerna som tumörceller har utvecklat för att fly undan immunsvaret, är hämning av den antigenpresenterande funktionen hos de dendritiska cellerna. Genom att låta de dendritiska cellerna ta upp tumörcellsmaterial och utmogna utanför patientens kropp, så tror man att den negativa influensen som tumören utövar på de dendritiska cellerna kan undvikas. Vaccinering av cancerpatienter med sådana dendritiska celler representerar en annan slags dendritcells-baserad immunoterapi strategi. Det är också viktigt att nämna att effektiviteten hos dendritcells-baserade vacciner påverkas av valet av presentations- och utmognadsmetod. I den andra delen av avhandlingen använde vi dendritcells-baserat vaccin för att behandla malignt gliom hos råtta. Vi testade olika antigenpresentations- och utmognadsmetoder. Alla undersökta parametrar visade dock att IFN-γ-modifierade tumörceller genererade ett bättre skydd mot gliom jämfört med dendritcells-baserade vaccinationer. Däremot så gav en kombination av IFN-γ-modifierade tumörceller och dendritceller en förbättrad immunterapi.

Sammanfattningsvis så har vi visat att immunterapi där man kombinerar IFN-γ modifierade tumörceller med dendritiska celler är mer fördelaktigt i behandling av råttor med experimentella hjärntumörer, än med enbart IFN-γ-modiferade tumörceller eller med enbart dendritiska celler. Resultaten i den här avhandlingen kan hjälpa till med att ytterligare utveckla anti-cancer vaccin.

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